Proteomic Analysis of Human Tendon and Ligament: Solubilization and Analysis of Insoluble Extracellular Matrix in Connective Tissues

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ABSTRACT: Connective tissues such as tendon, ligament and cartilage are mostly composed of extracellular matrix (ECM). These tissues are insoluble, mainly due to the highly cross-linked ECM proteins such as collagens. Difficulties obtaining suitable samples for mass spectrometric analysis render the application of modern proteomic technologies difficult. Complete solubilization of them would not only elucidate protein composition of normal tissues but also reveal pathophysiology of pathological tissues. Here we report complete solubilization of human
Achilles tendon and yellow ligament, which is achieved by chemical digestion combined with successive protease treatment including elastase. The digestion mixture was subjected to liquid chromatography-mass spectrometry. The low specificity of elastase was overcome by accurate mass analysis achieved using FT-ICR-MS. In addition to the detailed proteome of both tissues, we also quantitatively determine the major protein composition of samples, by measuring peak area of some characteristic peptides detected in tissue samples and in purified proteins. As a result, differences between human Achilles tendon and yellow ligament were elucidated at molecular level.

INTRODUCTION

Among various connective tissues, a tendon attaches muscle to bone, while a ligament is a fibrous tissue connecting bone with another bone. Reflecting their physiological functions, tendons are composed of collagenic fibers to transmit forces generated by muscles to bones, whereas ligaments are made of elastic fibers to hold structures. Ligamentum flavum (yellow ligament) is a ligament in spine, and their marked elasticity helps the vertebral column in resuming it after flexion. Many diseases and injuries occur in tendons and ligaments; subcutaneous rupture of tendons, rheumatoid arthritis, Dupuytren’s contracture, ossification of posterior longitudinal ligament, lumbar spinal canal stenosis due to hypertrophy of yellow ligament, and so on. As in many other diseases, proteomic analysis can contribute to the understanding of pathogenesis and the development of new treatments.

Recent development in mass spectrometry-based proteomics made it possible to assemble the proteome map of many human tissues. However, most of the connective tissues such as tendon and ligament are not included in these studies. Tendon and ligament are hypocellular and mainly composed of extracellular matrix (ECM), the major components being distinct types of
collagen and elastic fibers \(^1,^7\). The presence of abundant ECM proteins, especially elastin that is an insoluble protein \(^8,^9\), and extensive cross-links between the ECM molecules renders it difficult to prepare samples suitable for mass spectrometry-based proteomics. Although many studies have been reported on tendon and ligament with biomechanical, histological, genetic, and cell culture techniques \(^1,^10-^15\), there are few, if any, reports on detailed protein composition of them.

To characterize protein components in tendon and ligament, we first tried to establish a solubilization method suitable for human yellow ligament, which has been characterized by an exceptionally high content of elastin \(^10,^16\). By combining cyanogen bromide (CNBr) chemical cleavage with successive protease digestion including elastase, we could successfully solubilize the yellow ligament samples obtained from human subjects. The same digestion protocol was applied to Achilles tendon. Detailed proteomic analyses using liquid chromatography/mass spectrometry (LC/MS) were carried out with these samples. To characterize the difference between the two tissues quantitative analysis was also carried out. The results obtained showed not only the presence of elastin as major component in yellow ligament but also that of distinct collagen isoforms in yellow ligament and Achilles tendon.

**EXPERIMENTAL PROCEDURES**

**Materials**

Guanidine hydrochloride (Gu-HCl) was obtained from Sigma–Aldrich (St. Louis, MO), while formic acid (FA), CNBr, and lysyl endopeptidase (Lys-C, MS grade) were obtained from Wako Chemicals (Osaka, Japan). Urea was obtained from MP Biomedicals (Santa Ana, CA). Trypsin (sequence grade) was purchased from Promega (Tokyo, Japan), while elastase was from Elastin Products Company (Owensville, MO, USA). As for standard proteins used for quantitation,
collagen type I isolated from human skin (C5483), type III isolated from human placenta (C4407), and elastin isolated from human aorta (E6902) purchased from Sigma-Aldrich were used without further purification. All other chemicals and biochemicals were of highest grade commercially obtained.

**Subjects**

The human Achilles tendon samples were obtained from three patients (A1: 58 years, female, A2: 74 years, male, A3: 73 years, male) in the context of amputation due to tumor (Table 1). These tumors localized in ankle joint (A1), thigh (A2), or proximal lower leg (A3), and did not extend to Achilles tendon. On the other hand, the human yellow ligament samples were obtained from six patients (Y1: 71 years, female, Y2: 69 years, female, Y3: 65 years, female, Y4: 73 years, male, Y5: 78 years, female, Y6: 66 years, male) at the time of operation for lumbar spinal canal stenosis (Table 1). The Achilles tendon samples from two subjects (A1 and A2) and the yellow ligament samples from five subjects (Y1 through Y5) were used for proteomic analysis, while the Achilles tendon sample from one subject (A3) and the yellow ligament sample from one subject (Y6) were used for immunohistochemical analysis. This study was approved by the Ethics Committee of Tokushima University Hospital, and all the patients gave their informed consent. Tendon and ligament samples used for proteomic analysis were kept in cold phosphate buffer saline (PBS) for transportation. They were washed extensively in cold PBS to remove blood cells, wiped on paper towels, and kept frozen at -80 °C until further use.

**Sample Preparation**

The yellow ligament samples from two subjects (Y1 and Y2) and Achilles tendon samples from two subjects (A1 and A2) were subjected to the successive solubilization (Figure 1A). These samples were axially cut into 1-µm slices with cryotome (CM3050S, LEICA). Slices (6.5
mg wet weight) were extracted first in 350 µl of 4 M Gu-HCl in 50 mM Tris-HCl, pH 7.4, with stirring at 4 °C for 48 h to remove proteoglycans. The mixture was centrifuged at 14,000 × g for 60 min, and the resulting supernatant was dialyzed against a large volume distilled water at 4 °C to remove Gu-HCl and kept frozen until use (Gu-HCl fraction). The pellet was washed once with 350 µl of the same buffer and centrifuged again. The pellet was suspended in 1.5 ml of distilled water, and dialyzed against distilled water at 4 °C. The pellet was collected by centrifugation at 14,000 × g for 60 min at 4 °C, suspended in 40 µl of 100% FA, and incubated for 1 hour at 4 °C. To the solution, 2 mg of CNBr dissolved in 20 µl of 100% FA was added and the mixture was incubated at 30 °C under N\textsubscript{2} gas for 12 h. The same amount of CNBr was added and incubated for another 12 h. The reaction mixture was diluted tenfold with distilled water and lyophilized.

The freeze-dried sample was suspended in 80 µl of 8 M urea in 5 mM NH\textsubscript{4}HCO\textsubscript{3}, and mixed with 80 µl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 4% (w/v) SDS) containing 1% (w/v) DTT, and incubated for 5 min at 95 °C. Insoluble material was removed by centrifugation at 14,000 × g for 15 min at 20 °C. The supernatant was subjected to SDS-PAGE and in-gel digestion as described below (CNBr fraction). The whole CNBr treatment procedure was done under the hood, and the waste material contaminated with CNBr was treated with 5% sodium hypochlorite solution.

After removal of CNBr fraction, the resulting pellet was suspended and incubated for 15 min at 40 °C with 8 M urea and 200 mM NH\textsubscript{4}HCO\textsubscript{3} before reduction with 5 mM DTT at 56 °C for 60 min followed by alkylation with 10 mM iodoacetamide in the dark for 45 min at room temperature. The mixture was diluted with 200 mM NH\textsubscript{4}HCO\textsubscript{3} to make 4 M urea, and 50 µl of Lys-C (20 µg dissolved in 50 mM Tris-HCl, pH 8.5) was added and incubated overnight at 37
°C. The reaction mixture was diluted to make 2 M urea and digested with sequencing grade trypsin (20 µg dissolved in 100 µl of 50mM acetic acid) overnight at 37 °C. The reaction was terminated by adding 2.2 µl of FA. The mixture was centrifuged at 14,000 × g for 15 min at 15 °C, and the supernatant was saved for LC/MS analysis (Lys-C/trypsin fraction). The pellet was suspended in 100 µl of distilled water, and dried in a SpeedVac concentrator (SPD1010, Savant). The pellet was re-suspended in 30 µl of 50 mM Tris-HCl, pH8.5, and 30 µl of elastase solution (30 µg dissolved in 50 mM Tris-HCl, pH 8.5) was added and incubated for 24 hr at 37 °C. The reaction mixture was centrifuged at 14,000 × g for 15 min at 4 °C. At this stage almost no pellet was observed with visual inspection (Figure 1C). The supernatant (Elastase fraction) was subjected to LC/MS analysis as described below.

**Complete Solubilization in One Tube for Quantitative Analysis**

For quantitative analysis of the major component proteins, the freeze-dried slices obtained after Gu-HCl wash were subjected to successive digestion in a single tube (Figure 1B). The Achilles tendon samples from two subjects (A1 and A2) and the yellow ligament samples from five subjects (Y1 through Y5, Table 1) were cut into slices and extracted with Gu-HCl as described above. The pellets obtained were treated with CNBr as described above except that the treated samples were diluted with tenfold with distilled water and lyophilized without centrifugation. The lyophilized products were suspended in 30 µl of 50 mM Tris-HCl, pH8.5, and 60 µl elastase (1 µg/µl in 50 mM Tris-HCl, pH 8.5) was added and incubated for 12 hr at 37 °C. The same amount of elastase was added and incubated for another 12 hr at 37 °C. The suspension was concentrated in a SpeedVac avoiding complete dryness. The sample re-suspended in 8 M urea in 200 mM NH₄HCO₃ was reduced and alkylated as described above. The mixture was further digested successively with 40µg Lys-C dissolved in 50 µl of 50 mM
Tris-HCl, pH 8.5 and 40 µg trypsin dissolved in 100 µl 50 mM acetic acid as described above. The final urea concentration was 4 M for Lys-C digestion and 2 M for trypsin digestion. The digestion was terminated with 2.2 µl of FA. The digestion mixture was centrifuged at 14,000 × g for 15 min at 15 °C. The supernatant solution was desalted using a Bond Elute C18 cartridge (50 mg; Varian), and subjected to LC/MS. Little, if any, pellet was observed with visual inspection (Figure 1D).

Gel Electrophoresis and In-gel Digestion – Gu-HCl and CNBr fractions (Figure 1A) were subjected to SDS-PAGE. Two-thirds of the total Gu-HCl fraction was loaded onto an 8% SDS-polyacrylamide gel (Supplementary Figure S-1). One-fifth of the total CNBr fraction was loaded onto a 10% SDS-polyacrylamide gel (Supplementary Figure S-2). Gels were stained with Coomassie blue (Invitrogen). Gel lanes were excised for each of the samples and divided into 19 sections of approximately equal size (Supplementary Figures S-1 and S-2). Each slice was cut into 1-mm cubes prior to digestion. The gel pieces were placed in a 1.5-ml polypropylene tube, destained twice with 100 µl of 50% and 20% acetonitrile (ACN) in 25 mM NH₄HCO₃, and dehydrated with 100 µl of 100% ACN for 5 min. After removal of the ACN by aspiration, the gel pieces were dried in a SpeedVac for 15 min. The in-gel digestion with trypsin was carried out as described previously.¹⁷

Preparation of Standard Protein Digests

Human collagen type I, type III and elastin were used as standard. They were treated successively with CNBr, elastase, Lys-C and trypsin in a single tube without fractionation as described above. The digestion mixture was centrifuged, and the supernatant was desalted with a Bond Elute cartridge and used for LC/MS analysis.
**LC/MS analysis** – LC/MS analysis was performed on a hybrid linear ion trap (LTQ)-Fourier Transform Ion Cyclotron Resonance mass spectrometer (LTQ FT; Thermo Fisher; San Jose, CA). A nano HPLC system (Ultimate 3000, Dionex, Tokyo, Japan) was connected online to the self-constructed nano-spray interface as described previously. Chromatographic separation was performed on an Acclaim PepMap100 C18 column (3 μm particle, 75 μm i.d. × 150 mm, Thermo Fisher Scientific) with a Dionex μPrecolumn (5 μm particle, 300 μm i.d. × 5 mm) at 30 °C. As for gradient elution, solvent A consisted of 0.1% formic acid and 5% HPLC-grade ACN, whereas solvent B consisted of 0.1% formic acid and 95% HPLC-grade ACN. A sample was first loaded onto the trapping column and then washed with 0.1 % formic acid for 5 min. The gradient elution was programmed as follows: t.0 min, 0% B; t.31 min, 30% B; t.38 min, 60 % B; t.40 min, 95 % B; t.45 min, 95% B. The flow rate was 0.18 μl/min, and the injection volume was 6 μl, the samples being dissolved in 1% TFA. Data were collected in the information-dependent acquisition mode. The full MS was operated in the positive-ion mode at 25,000 mass resolution in the FT-CIR-cell (m/z 450-2,000). Selected-ion monitoring (SIM) spectra and tandem mass (MS/MS) spectra of the three most abundant ions were recorded. SIM spectra of 10 Th width were obtained in the FT-ICR-cell with 100,000 resolution. The selected precursors were subjected to the CID in the ion trap of the instrument with the isolation width set to 2 Th using a collision energy of 35%. The Q activation value for all MS/MS spectra was set to 0.250, whereas the activation time was set to 30 ms. The data obtained were processed with Mascot distiller software (Ver. 2.5.01, Matrix Science, London, UK). The peak lists produced were used for database search using MASCOT software (Ver. 2.5, Matrix Science, London, UK) searching the Swiss-Prot databases (2015.3) with a mass tolerance of ± 1 ppm for the precursor and 0.5 Da for fragment ions. The taxonomy was restricted to Homo sapiens containing 20,203 sequences.
The modifications considered were acetyl (N-term), carbamidomethyl (Cys), deamidation (Asn and Gln), and oxidation (Met, Lys and Pro). For the CNBr fractions, the conversion of Met to homocysteic acid was also considered. False discovery rate was set to 0.01 for each measurement. Spectral counts and MASCOT scores obtained for each individual measurement and average ± S.E. together with CV (%) for each tissue ($n = 2$) are summarized in Supplementary Tables (Supplementary Tables S-1 to S-4). For the statistical analysis, a value of 1 (spectral count) or a value of 30 (MASCOT score) was assigned for non-detected proteins. The reproducibility of the quantitative analysis was verified by measuring each sample and standards at least three times.

**Immunohistochemical Analysis** – To investigate the expressions of collagen type I, type III, elastin, and fibronectin in human yellow ligament and in Achilles tendon by immunohistochemistry, tissue samples obtained from a patient (Y6) and those from a patient (A3) were used. 10% formalin-fixed, paraffin-embedded tissue samples were cut in 3-µm thick sections on a microtome. The sections were first incubated at 4 °C overnight with rabbit anti-collagen I (ab138492; Abcam, Cambridge, UK) at a dilution of 1:1500, mouse anti-collagen III (C7805; Sigma-Aldrich) at a dilution of 1:40, mouse anti-elastin (E4013; Sigma-Aldrich) at a dilution of 1:500, or mouse anti-fibronectin (SC-52331; Santa Cruz Biotechnology, Dallas, TX) at a dilution of 1:100. They were further incubated at room temperature for 30 min with a secondary antibody (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA). Bound peroxidase was visualized by incubating with ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA), as a golden-brown reaction product.

**RESULTS**
Sample Solubilization

Typical procedures used for proteomic analysis of most tissues include solubilization with denaturants and/or detergents such as urea or SDS followed by digestion with trypsin and/or Lys-C. We first tried to solubilize and digest the human ligament samples according to the canonical procedures, but large insoluble material remained even after solubilization with high concentration of urea/SDS and prolonged protease treatment. This is not surprising; the ligaments are known to contain large amounts on insoluble elastin fibers. We then turned to chemical cleavage with CNBr that is one of the most widely used methods for protein analysis of collagenic tissues. The CNBr treatment released some proteins including collagen isoforms as expected, but the resulting pellets were still resistant to protease treatment (data not shown). We then tested several proteases, and found elastase digestion very promising. In fact, elastase has been shown to digest insoluble elastin. By combining these procedures we could successfully solubilize human yellow ligament; very little, if any, pellets were detected after successive digestion with CNBr, Lys-C, trypsin and elastase as described under Experimental Procedures (Figure 1A). It is evident that large amounts of insoluble materials remained after the digestion with trypsin and Lys-C in the presence of urea. When the same procedure was applied to human Achilles tendon, similar results were obtained. In the latter tissue, the digestion with Lys-C and trypsin in the presence of urea left small amounts of insoluble materials, which were successfully digested with elastase. In both cases, almost no pellets were observed with visual inspection (Figure 1C). The fractions obtained after each treatment were separately subjected to LC/MS analysis as described under Experimental Procedures (Figure 1A).

Proteomic Analysis of Gu-HCl Fractions of Achilles Tendon and Yellow Ligament
Proteins in Gu-HCl fraction were separated by one-dimensional SDS-PAGE (Supplementary Figure S-1), subjected to in-gel tryptic digestion, and analyzed by LC/MS as described under Experimental Procedures. Gu-HCl has been used to extract proteoglycans from connective tissues such as aorta. In Gu-HCl fraction, 455 proteins were identified in human Achilles tendon, while 337 proteins were identified in human yellow ligament (Supplementary Table S-1). Although about one-third of the proteins identified in both tissues were collagens, various proteoglycans, glycoproteins and ECM-associated proteins were detected (Figure 2, Table 2 and Supplementary Table S-1). Serum-derived proteins such as albumin, hemoglobin, and immunoglobulins were also detected in the fractions and in CNBr fractions. The close inspection of protein profiles of Gu-HCl fractions elucidated the similarity and difference between the two tissues (Supplementary Figure S-3). Decorin, biglycan, lumican were the major proteoglycans in both tissues. On the other hand, the compositions of other minor proteoglycans differ between the two. The amount of aggrecan was higher in yellow ligament than in Achilles tendon (Table 2). In addition to the proteoglycans, various glycoproteins were also identified in the Gu-HCl fractions (Supplementary Figure S-3). Contrary to the proteoglycans, the compositions of glycoproteins differ significantly between the two tissues. In Achilles tendon, cartilage oligomeric matrix protein (COMP) is the major glycoprotein in the tissue, while fibronectin (FN1) replaces the position in yellow ligament. These proteins play pivotal roles in the ECM formation/organization of connective tissues. Other proteins showing differential expression between the two tissues are lactoadherin (milk fat globule-EGF factor 8 protein, MFGE8), thrombospondins 1 and fibrinogens rich in yellow ligament. Since COMP belongs to the thrombospondin superfamily (thrombospondin 5), more than half of the glycoproteins are thrombospondins in Achilles tendon. It is noteworthy that fibronectin and lactoadherin, two cell
adhesion proteins, are abundant in yellow ligament. The latter has been shown to bind to elastin and connect smooth muscle to elastic fiber in arteries \(^{27}\). Other ECM-associated proteins were also identified in the fractions. The proteins showing large differences between the Gu-HCl fractions from the two tissues are apolipoproteins, chondroadherin, clusterin, dermatopontin, hyaluronan and proteoglycan link protein 1, metalloproteinase inhibitor 3 (TIMP3), and target of Nesh-SH3 found in the yellow ligament fraction (Table 2).

**Proteomic Analysis of CNBr Fractions**

Proteins in the CNBr fraction were separated by one-dimensional SDS-PAGE, in-gel digested, and analyzed by LC/MS as described under Experimental Procedures. In the CNBr fractions, 179 proteins were identified in human Achilles tendon, while 199 proteins were identified in human yellow ligament (Supplementary Table S-2). CNBr cleavage is one of the most commonly used methods for collagen analysis \(^{27}\). As expected, about two-thirds of proteins identified in both tissues were various types of collagens (Figure 2). Proteoglycans, glycoproteins and ECM-associated proteins were also detected and the compositions were similar to those detected in the Gu-HCl fraction, suggesting that they were carry over from the Gu-HCl fractions. However, it should be noted that the collagen isoforms detected in the two tissues are distinct: while the most abundant collagen isoforms are type I collagens (alpha1 and alpha 2 chains) in both tissues, significant amounts of collagen type III exist in yellow ligament (Table 3 and Supplementary Figure S-4).

**Proteins Identified in Lys-C/trypsin Fractions**

Peptides produced by Lys-C/trypsin digestion were directly subjected to LC/MS analysis. As is evident from the pie chart, most of the proteins identified in the fractions obtained from both tissues were collagens (Figure 2 and Table 4). While the major collagen isoforms in the Achilles
tendon were type I collagen, type III collagen is also rich in yellow ligament, reflecting the collagen compositions in the CNBr fractions (Table 4 and Supplementary Figure S-4). There were not many proteins detected in the fractions, suggesting that most of the proteins cleavable with Lys-C and trypsin have been either extracted by Gu-HCl or partially cleaved by CNBr.

**Proteins Identified in Elastase Fractions**

Peptides released by elastase digestion were analyzed by LC/MS as described under Experimental Procedures. Even after various treatments, a large insoluble material was observed in the yellow ligament sample, while the corresponding pellets were much smaller in the Achilles tendon sample. After the elastase digestion, no pellets were observed by visual inspection (Figure 1C). The insoluble material obtained after Lys-C/trypsin digestion disappeared almost completely after the elastase digestion, and this is more evident in the samples obtained from yellow ligament.

Major proteins identified in this fraction obtained from Achilles tendon were various isoforms of collagen, and elastin was detected as minor component (Figure 2 and Table 5). On the other hand, the most abundant protein detected in the fraction from yellow ligament was elastin (Figure 2 and Table 5). Various isoforms of collagen were also detected. It should be noted that elastin was identified only in the elastase fractions, suggesting that the insoluble elastin is resistant to chemical and protease digestion with CNBr and conventional proteases such as trypsin and Lys-C. Elastase digestion also released various types of collagens, but the isoform profiles are distinct from those observed in the previous fractions, suggesting that some of the collagen isoforms detected in the elastase fractions are resistant to CNBr cleavage and Lys-C/trypsin digestion (Supplementary Figure S-4). Close interactions of these isoforms with elastin may be the possible cause.
Quantitative Analysis of Major Protein Components

To gain more quantitative view of protein composition of the two tissues, the whole digestion procedure was done in a single tube without fractionation. We concentrated on the quantitation of the major collagen isoform, i.e., type I collagen, and elastin, since the high content of collagenic fibers in tendon and that of elastic fibers in ligament forms the major basis of physiological functions of these tissues. We also included type III collagen in the analysis, since the content of the isoform was remarkably high in yellow ligament (Supplementary Figure S-4). Standard proteins and all samples (2 Achilles tendon and 5 yellow ligament samples) were digested successively without fractionation as described under Experimental Procedures. Characteristic peptides were chosen for the three proteins, and standard curves were obtained for purified proteins using peak area (Supplementary Figures S-5 and S-6). Peptides used for the analysis are summarized in Supplementary Table S-5. The amino acid sequences of all peptides were confirmed by MS/MS analysis (data not shown). The absolute amounts of the proteins chosen were calculated per mg wet tissue. In tissue slices obtained from human Achilles tendon, the content of collagen type I was 211.7 ± 40.9 µg/mg tissue (Mean ± S.E.), while that of collagen type III was 15.3 ± 4.7µg/mg (Figure 3). No significant amount of elastin was detected. These results demonstrate the limitation of the whole digestion protocol in one tube. Namely, the presence of highly-abundant proteins such as type I collagen hinders the detection of minor proteins in the same sample. This may be due to the limited dynamic range of detection and/or due to the ion suppression effects. On the other hand, the content of collagen type I in human yellow ligament tissue was 48.3 ± 35.3 µg/mg tissue (Mean ± S.E.). The content of type III collagen was as high as that of type I collagen (47.0 ± 30.2µg/mg tissue). Elastin was clearly the
most abundant protein in the tissue (320.2 ± 66.0µg/mg tissue). The high content of collagen type III and the presence of collagen type I in yellow ligament should be noted.

**Immunohistochemical Analysis of Major Protein Components**

Since the presence of collagen type III in human yellow ligament is new, we have conducted immunohistochemistry of several major proteins in Achilles tendon and yellow ligament tissues. As shown in Fig. 4, the expression of collagen type I, type III, elastin, and fibronectin was clearly observed in a yellow ligament sample. However, the staining pattern of collagen type I is different from those of collagen type III, elastin and fibronectin. The latter shows more or less homogeneous staining except for a small area near the dorsal side, while the staining of collagen type I is stronger in the area near the dorsal and dural sides (Fig. 4). The staining with collagen type I antibody of human Achilles tendon also demonstrates the abundance of the protein in the tissue. On the other hand, the staining of elastin and that of collagen III were associated mainly with vessels and other surrounding tissues. These results confirm the presence of type I and type III collagens and fibronectin in human yellow ligament detected in the proteomic analysis.

**DISCUSSION**

Despite an increased interest in proteomic analysis of various human tissues, no detailed study on whole proteomic analysis of tendon and ligament has been reported. Tendon and ligament are hypocellular and mostly composed of ECM, the major components of which are distinct types of collagen and elastic fibers. In classical studies, the presence and amounts of these proteins are estimated from amino acid compositions and the presence of typical cross-linked amino acids such as desmosin found in elastin. Difficulties in application of the modern proteomic analytical techniques lies in the insoluble nature of these connective tissues. The insolubility arises from the abundant and highly cross-linked ECM proteins such as collagens and elastin.
We succeeded in complete solubilization of human Achilles tendon and yellow ligament, and performed detailed proteomic analyses of these tissues. The elastase digestion was especially indispensable in the solubilization of yellow ligament. Following the successive treatment with Gu-HCl extraction, cleavage with CNBr followed by Lys-C, trypsin and elastase digestion, we identified 502 and 378 proteins from human Achilles tendon and yellow ligament, respectively. The detailed proteomic analysis demonstrated the differences in the composition of glycoproteins, proteoglycans, ECM-associated proteins and collagen isoforms between the two tissues.

**Comparison of Major Proteins Detected in Various Fractions Obtained from Achilles Tendon and Yellow Ligament**

Protein compositions of various fractions obtained from the two tissues are summarized in Figure 2. As is evident from the pie charts, collagen isoforms are the major protein family in all fractions except for the elastase fraction of yellow ligament. About one-thirds are collagens in the Gu-HCl fractions, while more than two-thirds are collagen isoforms in the CNBr fractions from both tissues. These results suggest that some of collagen isoforms remain soluble, and can be extracted by Gu-HCl, but most of collagens can be released only by CNBr cleavage. At this stage, only elastin and collagens except for some minor proteins remain insoluble. The successive digestion with Lys-C and trypsin released part of the collagen isoforms, but elastin remained insoluble at this stage, demonstrating that elastin is resistant to various treatments including chemical cleavage with CNBr. A small amount of elastin was also detected in the elastase fraction from Achilles tendon. On the other hand, no significant signal of elastin-derived peptide was observed in the quantitative analysis using unfractionated samples as described above. Although the whole Achilles tendon was stained with anti-elastin antibody, the
strong staining was associated mainly with vessels and surrounding tissues, suggesting the actual elastin content in Achilles tendon is rather low. Interestingly, distinct collagen isoforms were detected in the elastase fractions from both tissues, suggesting that some of the collagen isoforms may form insoluble matrix with elastin, and elastase digests not only elastin but also various collagen isoforms.

Quantitative analysis established the content of elastin and that of several major collagen isoforms in the two tissues. This is the first report which identified elastin and clarified types of collagen in these human connective tissues with distinct functional differences using proteomic analysis with LC/MS. The results not only confirm the knowledge described in the classic textbooks but also gave the more quantitative data on the contents of two major proteins in tendons and ligaments, i.e., elastin and collagen. The contents of elastin in Achilles tendon determined in the present study seem lower than those reported in the classic literature 4. This may be due to the limitation of the quantitation method employed in the present study, i.e., the lack of basic amino acids in the proteins hinders the efficient ionization of peptides, and the matrix effects may also lower the ionization. On the other hand, the immunohistochemical analysis demonstrated the staining for elastin is mainly associated with vessel and surrounding tissues in Achilles tendon (Figure 4). The way of sample preparation may well affect the quantitation results of the protein in the tissue.

**Collagen Isoforms Expressed in Achilles Tendon and Yellow Ligament**

The present analysis gave detailed pictures on the presence of various collagen isoforms in the two tissues. These isoforms were identified with at least one unique peptide and the relationship between the various fractions are summarized (Supplementary Table S6). The collagen isoforms I alpha1 and I alpha2 form triple helix consisting of two I alpha1 chains and one I alpha2 chain
The contents of these isoforms in Achilles tendon are roughly reflected in the semi-quantitative data based on the spectral counts. On the other hand, the deviation from the two-to-one ratio in various fractions obtained from yellow ligament was observed, suggesting that some of the two collagen isoforms may not form the classical triple helix in the tissue. The collagen isoform III, which form another triple helix consisting of homo trimer, is the one of the three major collagen isoforms in both tissues. The amount of type III collagen is about the same as that of type I alpha1 in various fractions obtained from yellow ligament, while that of type III collagen is around 10% of that of type I alpha1 in the four fractions obtained from Achilles tendon. The quantitative analysis showed similar results. Furthermore, the immunohistochemistry showed intense staining in the whole tissue and colocalization with elastin (Fig. 4). Therefore, type III collagen is clearly one of the major collagen isoforms in yellow ligament.

Type III collagen, initially identified in human skin, are found in embryonic and elastic tissues. It forms thinner and more flexible fibers than type I collagen. It is, therefore, reasonable to find high contents of type III collagen in yellow ligament. On the other hand, type III collagen has been suggested to be involved in wound healing and appear increased in hypertrophic scars. It is possible that the high content of type III collagen observed in the present study is due to the fact that the yellow ligament specimens used in this study were obtained from lumbar spinal canal stenosis patients: a disease caused by trophic changes (thickening) of yellow ligament. The high content of fibronectin, which is a well-known player in the healing skin and suggested to form primary matrix with plasma fibrin, may also support the view. The high content of fibrin in the yellow ligament samples is also notable. The work on the relationship between the hypertrophy of yellow ligament and the content of collagen isoforms is now in progress.
Collagen isoforms can be classified based on the structural characteristics \(^{29, 34, 35}\). The classification of collagen isoforms revealed clear differences in minor isoforms expressed in the two tissues (Supplementary Figure S-4). Of network-forming collagens, type VIII collagens, which is known to form triple helices consisting of two VIII alpha1 chains and one VIII alpha2 chain ([\(\alpha_1(\text{VIII})_2\alpha_2(\text{VIII})\)]) are identified in yellow ligament. On the other hand, one of the isoforms belonging to the fibril-associated collagens (FACITs), the collagen XII is rich in Achilles tendon. Collagen VI, a microfibrillar collagen, shows also similar isoform compositions. Since the length of VI alpha 3 is three times longer than those of alpha1 and alpha 2, the protein amounts based on the spectral count corresponds very well to the presence of a hetero-trimer. These compositions are similar in the various fractions except for the elastase fraction. Minor isoforms such as collagen VII and XXII are detected only in the elastase fractions. These results may suggest that there exist strong interactions of these collagen isoforms with elastin.

**Proteoglycans, Glycoproteins and ECM-associated Proteins in Connective Tissues**

To our knowledge, the present work is the first detailed proteomic study on the connective tissues including the insoluble proteins. The Gu-HCl extraction, on the contrary, has been used to extract proteoglycans from connective tissues \(^{25}\). In fact, proteomic analyses of the Gu-HCl extracts from human aorta and human and mouse cartilage have been published previously \(^{36, 37-39}\). It is, therefore, possible to compare the protein compositions of proteoglycans and other ECM-associated proteins of four connective tissues (Supplementary Figure S-3). As for glycoproteins, the major glycoprotein identified in human aorta and yellow ligament is fibronectin. On the other hand, cartilage oligomeric matrix protein (COMP) is the major glycoprotein in Achilles tendon and cartilage. Although the COMP protein is also expressed in
ligament as has been demonstrated by western blot and metabolic labeling \(^{40}\), it is almost lacking in aorta. These results suggest that fibronectin plays an important role in the elastic fiber-cell interactions, whereas the COMP protein may be associated with collagenic fibers. The high expression of thrombospondin 1/4 in these tissues except in aorta is also noted. Lactoadherin (MFGE8) seems to be almost ubiquitously expressed in the four connective tissues.

As for proteoglycans, decorin, biglycan, and lumican are expressed in the four connective tissues, although the relative contents differ significantly depending on the tissue. However, the high expression of HSPG2 (also called as perlecan or basement membrane-specific heparan sulfate proteoglycan core protein) is observed in aorta and cartilage, while that of versican was observed only in aorta. On the other hand, aggrecan was highly expressed in yellow ligament, aorta and cartilage, while the protein is a minor component in Achilles tendon. These results demonstrate distinct protein expression profiles in the four tissues, but also show some resemblance between ligament and aorta, two connective tissues rich in elastic fibers.

Other ECM-associated proteins show similar ubiquitous expression of some proteins and tissue-specific expression of other proteins. For example, PRELP (prolargin) is almost ubiquitously expressed in the four connective tissues, although the expression level in cartilage is low. PRELP, which is known to interact with collagen I in tendon and collagen II in cartilage, has been shown to interact with the basement membrane protein HSPG2 (perlecan) anchoring basement membranes to the underlying connective tissues \(^{41}\). As mentioned above, HSPG2 is highly expressed in aorta and cartilage but not in Achilles tendon and yellow ligament. These results suggest that the binding partner of PRELP may be different in the latter two tissues. The high expression of Cartilage intermediate layer protein 1 (CILP1) in Achilles tendon and in yellow ligament is also noted. As the name suggests CILP was first characterized in cartilage \(^{42}\).
and detected in human and mouse cartilage by recent proteomic analyses. In contrast, matrilins (MATN1 and 3), which connect collagen fibrils to other ECM proteins such as aggrecan, are clearly one of the major components in cartilage, but not expressed in other three connective tissues. Altogether these results show clear differences in the molecular organizations of extracellular matrix in the four connective tissues whose proteome have been analyzed in depth.

In summary, CNBr and three proteases, trypsin, Lys-C and elastase, were found necessary for solubilization of human tendon and ligament. Especially the elastase digestion was essential for the solubilization and complete digestion of elastin-containing tissues. Elastase has been shown to work with insoluble elastic fibers, whereas trypsin is ineffective on them. Interestingly, elastase cleaved insoluble collagen isoforms associated with elastin. This may have implications in the pathology of connective tissues, in which collagenolysis and elastolysis by MMPs and other proteases play important roles. Although the collagen I cleavage by neutrophil elastase has been demonstrated, the present work is the first demonstration of cleavage of collagen isoforms by pancreatic elastase. Finally, the present solubilization procedures may well be applicable to other connective tissues such as vessels, cartilage, skin and bone, which contain abundant extracellular matrix proteins.

Supporting Information.

The supporting information is available free of charge at ACS website

http://pubs.acs.org:
Figure S-1. SDS-PAGE of Gu-HCl fractions from human Achilles tendon and yellow ligament. Figure S-2. SDS-PAGE of CNBr fractions from human Achilles tendon and yellow ligament. Figure S-3. Proteins identified in Gu-HCl fractions from Achilles tendon and yellow ligament were compared with those from human aorta and mouse cartilage. Figure S-4. Types of collagens identified in human Achilles tendon and yellow ligament. Figure S-5. Extracted ion chromatograms and MS spectra of peptides used for quantitation. Figure S-6. Standard curves for peptides used for quantitation of two collagen isoforms and elastin. (PDF)

Table S-1. Extracellular matrix and associated proteins identified in Gu-HCl fraction. Table S-2. Extracellular matrix and associated proteins identified in CNBr fraction. Table S-3. Proteins identified in LysC-trypsin fraction. Table S-4. Proteins identified in elastase fraction. Table S-5. Peptides used for quantitative analysis. Table S-6. Number of unique peptides used for identification of collagen isoforms. (XLSX)

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Author contributions: NS, TT, YG, NY, KS and HT designed research; NS, TT and YG performed research; YG, HK, KH, TS, SK and KS contributed samples; NS and HT wrote the paper. All authors have given approval to the final version of the manuscript.

These authors contributed equally.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ECM, extracellular matrix; CNBr, cyanogen bromide; Gu-HCl, guanidine hydrochloride; FA, formic acid; ACN, acetonitrile; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; LC, liquid chromatography; MS, mass spectrometry; Lys-C, lysyl endopeptidase; COMP, cartilage oligomeric matrix protein.
REFERENCES


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Analytical biochemistry 278, 99-105.


Table 1. Clinical information of patients whose tissue were analyzed.

<table>
<thead>
<tr>
<th>Sample reference</th>
<th>Gender</th>
<th>Age</th>
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<th>Disease</th>
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<tbody>
<tr>
<td>A1</td>
<td>F</td>
<td>58</td>
<td>Achilles tendon</td>
<td>Malignant tumor of ankle joint (^a)</td>
</tr>
<tr>
<td>A2</td>
<td>M</td>
<td>74</td>
<td>Achilles tendon</td>
<td>Malignant tumor of thigh (^b)</td>
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<tr>
<td>Y1</td>
<td>F</td>
<td>71</td>
<td>Yellow ligament</td>
<td>Lumbar spinal canal stenosis</td>
</tr>
<tr>
<td>Y2</td>
<td>F</td>
<td>69</td>
<td>Yellow ligament</td>
<td>Lumbar spinal canal stenosis</td>
</tr>
<tr>
<td>Y3</td>
<td>F</td>
<td>65</td>
<td>Yellow ligament</td>
<td>Lumbar spinal canal stenosis</td>
</tr>
<tr>
<td>Y4</td>
<td>M</td>
<td>73</td>
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</tr>
<tr>
<td>Y5</td>
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<td>Lumbar spinal canal stenosis</td>
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<td>A3</td>
<td>M</td>
<td>73</td>
<td>Achilles tendon</td>
<td>Malignant tumor of proximal lower leg (^c)</td>
</tr>
<tr>
<td>Y6</td>
<td>M</td>
<td>66</td>
<td>Yellow ligament</td>
<td>Lumbar spinal canal stenosis</td>
</tr>
</tbody>
</table>

\( ^a\)The tumor localized in ankle joint, and did not extend to Achilles tendon.

\( ^b\)The tumor localized in thigh, and did not extend to Achilles tendon.

\( ^c\)The tumor localized in proximal lower leg, and did not extend to Achilles tendon.
Table 2. Major ECM and ECM-associated proteins identified in Gu-HCl fractions.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>UniProt Accession No.</th>
<th>MW (kDa)</th>
<th>Spectral count (Mean ± S.E.)</th>
<th>p value</th>
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<tbody>
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<td></td>
<td>Yellow ligament</td>
<td>Achilles tendon</td>
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<tr>
<td>Collagens</td>
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<td></td>
<td></td>
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<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_HUMAN</td>
<td>139</td>
<td>535 ± 60</td>
<td>1016 ± 324</td>
</tr>
<tr>
<td>Collagen alpha-2(I) chain</td>
<td>CO1A2_HUMAN</td>
<td>129</td>
<td>383 ± 8</td>
<td>571 ± 60</td>
</tr>
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<td>Collagen alpha-1(II) chain</td>
<td>CO2A1_HUMAN</td>
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<td>100 ± 17</td>
<td>N.D.</td>
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<td>Collagen alpha-1(III) chain</td>
<td>CO3A1_HUMAN</td>
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<td>518 ± 45</td>
<td>275 ± 163</td>
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<td>Collagen alpha-1(V) chain</td>
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<td>62 ± 27</td>
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<td>Glycoprotein</td>
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<td></td>
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<td>Sample Type</td>
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<td>Ctrl Mean ± StD</td>
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<td>Annexin A5</td>
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<td>Cartilage intermediate layer protein 2</td>
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</tr>
<tr>
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<td>Dermatopontin</td>
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<td>Extracellular superoxide dismutase [Cu-Zn]</td>
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<td>0 ± 0</td>
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</table>

Achilles tendon samples obtained from two subjects and yellow ligament samples obtained from two subjects were subjected to Gu-HCl extraction and gel-enhanced LC/MS analysis. Each sample was analyzed twice. Values are shown as mean ± S.E. of two samples. N.D.: not detected.
Table 3. Major ECM and ECM-associated proteins identified in CNBr fractions.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>UniProt Accession No.</th>
<th>MW (kDa)</th>
<th>Spectral count (Mean ± S.E.)</th>
<th>p value</th>
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<tr>
<td></td>
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<td>Yellow ligament</td>
<td>Achilles tendon</td>
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<tr>
<td>Collagens</td>
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</tr>
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<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_HUMAN</td>
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<td>142 ± 94</td>
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<td>823 ± 187</td>
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<td>80 ± 5</td>
<td>14 ± 7</td>
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<tr>
<td>Collagen alpha-2(V) chain</td>
<td>CO5A2_HUMAN</td>
<td>145</td>
<td>66 ± 5</td>
<td>3 ± 3</td>
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<td>Collagen alpha-1(VI) chain</td>
<td>CO6A1_HUMAN</td>
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<td>82 ± 14</td>
<td>107 ± 11</td>
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<tr>
<td>Collagen alpha-2(VI) chain</td>
<td>CO6A2_HUMAN</td>
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<td>45 ± 6</td>
<td>63 ± 5</td>
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<td>Collagen alpha-3(VI) chain</td>
<td>CO6A3_HUMAN</td>
<td>343</td>
<td>238 ± 22</td>
<td>284 ± 1</td>
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<tr>
<td>Collagen alpha-1(VIII) chain</td>
<td>CO8A1_HUMAN</td>
<td>73</td>
<td>31 ± 2</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Collagen alpha-2(VIII) chain</td>
<td>CO8A2_HUMAN</td>
<td>67</td>
<td>5 ± 2</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Collagen alpha-1(XI) chain</td>
<td>COBA1_HUMAN</td>
<td>181</td>
<td>13 ± 7</td>
<td>25 ± 1</td>
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<tr>
<td>Collagen alpha-2(XI) chain</td>
<td>COBA2_HUMAN</td>
<td>172</td>
<td>0 ± 0</td>
<td>6 ± 2</td>
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<tr>
<td>Collagen alpha-1(XII) chain</td>
<td>COCA1_HUMAN</td>
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<td>9 ± 0</td>
<td>69 ± 6</td>
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<td>Proteoglycan</td>
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<td></td>
</tr>
<tr>
<td>Aggrecan core protein</td>
<td>PGCA_HUMAN</td>
<td>250</td>
<td>7 ± 7</td>
<td>N.D.</td>
</tr>
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<td>HSPG2 (Perlecan)</td>
<td>PGBM_HUMAN</td>
<td>469</td>
<td>29 ± 24</td>
<td>2 ± 1</td>
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<td>Biglycan</td>
<td>PGS1_HUMAN</td>
<td>42</td>
<td>45 ± 5</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>Decorin</td>
<td>PGS2_HUMAN</td>
<td>40</td>
<td>59 ± 6</td>
<td>54 ± 12</td>
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<tr>
<td>Fibromodulin</td>
<td>FMOG_HUMAN</td>
<td>43</td>
<td>5 ± 1</td>
<td>27 ± 8</td>
</tr>
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<td>Lumican</td>
<td>LUM_HUMAN</td>
<td>38</td>
<td>33 ± 3</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Mimecan</td>
<td>MIME_HUMAN</td>
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<td>19 ± 2</td>
<td>7 ± 2</td>
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<td>Glycoprotein</td>
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<td>COMP_HUMAN</td>
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<td>48 ± 17</td>
<td>110 ± 0</td>
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<td>protein</td>
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<td>Fibrillin-1</td>
<td>FBN1_HUMAN</td>
<td>312</td>
<td>50 ± 21</td>
<td>36 ± 1</td>
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<tr>
<td>Fibrinogen gamma chain</td>
<td>FIBG_HUMAN</td>
<td>51</td>
<td>12 ± 10</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>FINC_HUMAN</td>
<td>262</td>
<td>188 ± 70</td>
<td>19 ± 5</td>
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<td>Fibulin-1</td>
<td>FBLN1_HUMAN</td>
<td>77</td>
<td>15 ± 1</td>
<td>12 ± 7</td>
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<td>Periostin</td>
<td>POSTN_HUMAN</td>
<td>93</td>
<td>15 ± 2</td>
<td>1 ± 0</td>
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<td>Thrombospondin-1</td>
<td>TSP1_HUMAN</td>
<td>129</td>
<td>46 ± 9</td>
<td>8 ± 2</td>
</tr>
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<td>Thrombospondin-4</td>
<td>TSP4_HUMAN</td>
<td>106</td>
<td>10 ± 2</td>
<td>75 ± 33</td>
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<td>Vitronectin</td>
<td>VTNC_HUMAN</td>
<td>54</td>
<td>25 ± 5</td>
<td>22 ± 3</td>
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<tr>
<td>ECM-associated proteins</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin, alpha skeletal muscle</td>
<td>ACTS_HUMAN</td>
<td>42</td>
<td>2 ± 2</td>
<td>42 ± 34</td>
</tr>
<tr>
<td>Actin, aortic smooth muscle</td>
<td>ACTA_HUMAN</td>
<td>42</td>
<td>2 ± 2</td>
<td>30 ± 22</td>
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<tr>
<td>Actin, cytoplasmic 1</td>
<td>ACTB_HUMAN</td>
<td>42</td>
<td>7 ± 4</td>
<td>21 ± 6</td>
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<tr>
<td>Actin, alpha cardiac muscle 1</td>
<td>ACTC_HUMAN</td>
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</tr>
<tr>
<td>Protein Name</td>
<td>Gene Symbol</td>
<td>Unit 1</td>
<td>Unit 2</td>
<td>p-Value</td>
</tr>
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<td>--------------------------------------------</td>
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</tr>
<tr>
<td>Annexin A1</td>
<td>ANXA1_HUMAN</td>
<td>39</td>
<td>0 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Annexin A2</td>
<td>ANXA2_HUMAN</td>
<td>39</td>
<td>4 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Annexin A5</td>
<td>ANXA5_HUMAN</td>
<td>36</td>
<td>6 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>APOA1_HUMAN</td>
<td>31</td>
<td>12 ± 3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Apolipoprotein A-IV</td>
<td>APOA4_HUMAN</td>
<td>45</td>
<td>12 ± 7</td>
<td>N.D.</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>APOE_HUMAN</td>
<td>36</td>
<td>12 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Asporin</td>
<td>ASPN_HUMAN</td>
<td>43</td>
<td>34 ± 16</td>
<td>20 ± 1</td>
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<tr>
<td>Carboxypeptidase B2</td>
<td>CBPB2_HUMAN</td>
<td>48</td>
<td>7 ± 4</td>
<td>N.D.</td>
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<tr>
<td>Cartilage intermediate layer protein 1</td>
<td>CILP1_HUMAN</td>
<td>132</td>
<td>92 ± 24</td>
<td>105 ± 25</td>
</tr>
<tr>
<td>Cartilage intermediate layer protein 2</td>
<td>CILP2_HUMAN</td>
<td>126</td>
<td>30 ± 16</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>Chondroadherin</td>
<td>CHAD_HUMAN</td>
<td>40</td>
<td>51 ± 16</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Clusterin</td>
<td>CLUS_HUMAN</td>
<td>52</td>
<td>42 ± 5</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Dermatopontin</td>
<td>DERM_HUMAN</td>
<td>24</td>
<td>7 ± 0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Hyaluronan and proteoglycan link protein 1</td>
<td>HPLN1_HUMAN</td>
<td>40</td>
<td>6 ± 5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Latent-transforming growth factor beta-binding protein 2</td>
<td>LTBP2_HUMAN</td>
<td>195</td>
<td>16 ± 1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Metalloproteinase inhibitor 3</td>
<td>TIMP3_HUMAN</td>
<td>24</td>
<td>30 ± 13</td>
<td>18 ± 14</td>
</tr>
<tr>
<td>Myocilin</td>
<td>MYOC_HUMAN</td>
<td>57</td>
<td>N.D.</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>Prolargin</td>
<td>PRELP_HUMAN</td>
<td>44</td>
<td>103 ± 12</td>
<td>89 ± 15</td>
</tr>
<tr>
<td>Serum amyloid P-component</td>
<td>SAMP_HUMAN</td>
<td>25</td>
<td>65 ± 2</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>Stromelysin-1</td>
<td>MMP3_HUMAN</td>
<td>54</td>
<td>0 ± 0</td>
<td>14 ± 12</td>
</tr>
<tr>
<td>Transforming growth factor-beta-induced protein ig-h3</td>
<td>BGH3_HUMAN</td>
<td>75</td>
<td>9 ± 4</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

Achilles tendon samples obtained from two subjects and yellow ligament samples obtained from two subjects were subjected to CNBr digestion and gel-enhanced LC/MS analysis. Each sample was analyzed twice. Values are shown as mean ± S.E. of two samples. N.D.: not detected.
Table 4. Proteins identified in LysC-Trypsin fractions.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>UniProt Accession No.</th>
<th>MW (kDa)</th>
<th>Spectral count (Mean ± S.E.)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yellow ligament</td>
<td>Achilles tendon</td>
</tr>
<tr>
<td>Collagens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_HUMAN</td>
<td>139</td>
<td>44 ± 1</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>Collagen alpha-2(I) chain</td>
<td>CO1A2_HUMAN</td>
<td>129</td>
<td>9 ± 1</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Collagen alpha-1(II) chain</td>
<td>CO2A1_HUMAN</td>
<td>142</td>
<td>1 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Collagen alpha-1(III) chain</td>
<td>CO3A1_HUMAN</td>
<td>138</td>
<td>31 ± 3</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Collagen alpha-1(VIII) chain</td>
<td>CO8A1_HUMAN</td>
<td>73</td>
<td>1 ± 0</td>
<td>N.D.</td>
</tr>
<tr>
<td>ECM-associated proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase inhibitor 3</td>
<td>TIMP3_HUMAN</td>
<td>24</td>
<td>1 ± 0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Achilles tendon samples obtained from two subjects and yellow ligament samples obtained from two subjects were subjected to successive digestion as described under Experimental Procedures. Peptides released by Lys-C/trypsin digestion were directly injected to LC/MS. Each sample was analyzed twice. Values are shown as mean ± S.E. of two samples. N.D.: not detected.
Table 5. Proteins identified in elastase fractions.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>UniProt Accession No.</th>
<th>MW (kDa)</th>
<th>Spectral count (Mean ± S.E.)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yellow ligament</td>
<td>Achilles tendon</td>
</tr>
<tr>
<td>Elastin</td>
<td>ELN_HUMAN</td>
<td>68</td>
<td>141 ± 7</td>
<td>9 ± 8</td>
</tr>
<tr>
<td>Collagens</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_HUMAN</td>
<td>139</td>
<td>22 ± 4</td>
<td>54 ± 19</td>
</tr>
<tr>
<td>Collagen alpha-2(I) chain</td>
<td>CO1A2_HUMAN</td>
<td>129</td>
<td>12 ± 6</td>
<td>27 ± 19</td>
</tr>
<tr>
<td>Collagen alpha-1(III) chain</td>
<td>CO3A1_HUMAN</td>
<td>138</td>
<td>16 ± 8</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Collagen alpha-5(IV) chain</td>
<td>CO4A5_HUMAN</td>
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<td>N.D.</td>
<td>2 ± 0</td>
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<td>Collagen alpha-1(VII) chain</td>
<td>CO7A1_HUMAN</td>
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<td>2 ± 1</td>
<td>0 ± 0</td>
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<tr>
<td>Collagen alpha-1(VIII) chain</td>
<td>CO8A1_HUMAN</td>
<td>73</td>
<td>2 ± 2</td>
<td>N.D.</td>
</tr>
<tr>
<td>Collagen alpha-2(VIII) chain</td>
<td>CO8A2_HUMAN</td>
<td>67</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Others</td>
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<td></td>
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<td>Chymotrypsin-like elastase family</td>
<td>CELA1_HUMAN</td>
<td>28</td>
<td>N.D.</td>
<td>3 ± 2</td>
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<tr>
<td>member 1</td>
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<tr>
<td>Chymotrypsin-like elastase family</td>
<td>CEL2A_HUMAN</td>
<td>29</td>
<td>N.D.</td>
<td>1 ± 1</td>
</tr>
<tr>
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<td>Chymotrypsin-like elastase family</td>
<td>CEL2B_HUMAN</td>
<td>29</td>
<td>N.D.</td>
<td>5 ± 2</td>
</tr>
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<td>member 2B</td>
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<td>Epsin-1</td>
<td>EPN1_HUMAN</td>
<td>60</td>
<td>N.D.</td>
<td>0 ± 0</td>
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</table>

Achilles tendon samples obtained from two subjects and yellow ligament samples obtained from two subjects were subjected to successive digestion as described under Experimental Procedures. Peptides released by elastase digestion were directly injected to LC/MS. Each sample was analyzed twice. Values are shown as mean ± S.E. of two samples. N.D.: not detected.
For TOC only

Table of Contents Graphic

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Figure 1. A schematic diagram of two solubilization procedures of human tendon and ligament. (A) Stepwise solubilization for qualitative analysis. Tissue slices were first extracted with Gu-HCl, and the resulting pellet fractions were digested with CNBr. The pellet fractions were then digested with Lys-C and trypsin. The insoluble material was then digested with elastase. Almost no pellets were observed after elastase digestion (C). The supernatant fractions after each treatment were separately analyzed by gel-enhanced LC/MS (Gu-HCl and CNBr fractions) or by LC/MS (Lys-C/trypsin and elastase fractions). (B) Solubilization in one tube. After Gu-HCl extraction, the resulting pellets were digested successively with CNBr and various proteases without centrifugation. The resulting digests were analyzed directly by LC/MS analysis to obtain quantitative data. Almost no pellets were observed after successive digestion in one tube (D).
Figure 2. Functional classification of proteins identified in fractions from human Achilles tendon and yellow ligament. Proteins identified in each fraction obtained by successive digestion were classified based on their functions. The amounts of proteins were estimated from their spectral counts.

105x148mm (300 x 300 DPI)
Figure 3. Quantitative analysis of major proteins identified in human Achilles tendon and yellow ligament. The quantitation of elastin and two collagen isoforms was performed as described under Experimental Procedures. Protein contents are in µg protein/ mg wet tissue. Elastin and two major collagen isoforms in Achilles tendon (open bar) and yellow ligament (closed bar). N.D., not detected.
Figure 4. Immunohistochemical analysis of major proteins expressed in human Achilles tendon and yellow ligament. Tissue sections obtained from human subjects (Achilles tendon from patient A3 and yellow ligament from patient Y6) were stained with antibodies against collagen type I, type III, elastin and fibronectin as described under Experimental Procedures (magnification ×10 (left) and ×100 (enlarged, right)).

210x297mm (300 x 300 DPI)